Potential Application of Vanadium Probes for Biological X-ray Microscopy

M.M. Moronne, D.J. Hamamoto, G. Meigs, L. E. Johnson, G. P. Denbeaux, and W. Meyer-Ilse

Lawrence Berkeley National Laboratory, Berkeley, CA, USA

Abstract. Soft x-ray microscopy is now routinely capable of imaging biological specimens with resolutions that are five times better than the best visible light microscopes (≤ 50 nm). However, for biological labeling the only options developed for x-ray microscopy have been silver enhanced gold probes that can be used with both scanning and wide field CCD microscopes, such as XM-1 at the Advanced Light Source (ALS), and luminescent lanthanide probes that necessitate a scanning microscope (SXM). To add to the arsenal of useful x-ray biological probes, we have begun the development of labels that rely on the L-edge absorption lines of vanadium. Vanadium is especially attractive as a biological contrast reagent because it has two strong absorption lines at energies that range from ~512 to 525 eV just below oxygen K-edge which make it an ideal material for imaging in the water window. In this report, we present our initial findings on the application of vanadium for biological labeling. Fixed NIH 3T3 cells grown on silicon nitride windows were incubated with vanadyl sulfate and in some cases basified with triethylamine. After vanadium treatment of the cells, they were thoroughly rinsed and then imaged using XM-1 above and below the vanadium 516 eV resonance. Vanadium staining was clearly visible around and in the cells. These findings suggest that bioconjugated vanadium clusters could provide sufficient x-ray contrast to be used as biological probes.

INTRODUCTION

In the past two decades, cell biology has been revolutionized by the development of fluorescent labels that can accurately target specific biological molecules and report the spatial distribution of cell components ranging from the cytoskeleton to nuclear transcription sites. In addition, the wide variety of emission colors makes it possible to perform simultaneous labeling of distinct cell proteins and to determine their geometric proximity. This information can be especially useful in identifying and quantifying possible functional interactions between specific cell proteins. However, conventional light microscopy using fluorescent probes is limited in resolution to about 200 nm. Although of great utility, in many instances this size scale is simply too large given that the dimensions of most proteins range from 1 to 10 nm.

The most direct approach to increase resolution beyond the visible light limit is to use techniques that can operate at shorter wavelengths. In recent years, soft x-ray microscopy (λ = 2 to 5 nm) has achieved resolutions close to 30 nm [1]. Future improvements are expected to give resolutions approaching 10 nm. However, despite the improved resolution of x-ray microscopes, and the high quality morphological

detail they can provide, there has been a lack of molecular probes capable of localizing specific cell proteins and other important subcellular targets. To circumvent this problem, labeling agents are needed that can specifically operate using x-ray illumination. In this regard, two approaches have been taken including silver enhanced gold labeling [2], and scanning luminescence x-ray microscopy (SLXM, [3, 4, 5]).

In this report, we describe the first results of soft x-ray contrast enhancement based on vanadium labeling. We show that the vanadium L-edges provide sufficiently narrow resonances, strong x-ray absorption, and favorably positioned water window energies (~512-525 eV) that make it an ideal candidate for biological x-ray labeling. Of particular importance, is the fact that vanadium based probes would be fully compatible with silver enhanced gold labeling making it possible to perform high resolution co-localization studies using CCD based x-ray imaging microscopes such as XM-1 at the Advanced Light Source (ALS) or the similar microscope at BESSY.

METHODS

Vanadium Spectroscopy. Vanadium $L_{2,3}$ edge absorption lines were examined using both dry precipitated samples on a silicon nitride window with the ALS scanning transmission microscope (STXM) on beamline 7.0.1 and in aqueous 1 M $VOSO_4$ solutions sandwiched between silicon nitride windows using the CCD imaging microscope XM-1. The latter instrument provides lower spectral resolution than the undulator driven STXM and there are some differences in the wavelength calibrations, but the results are quite comparable in demonstrating the narrowness of $L_{2,3}$ resonances. The vanadium hydrated oxide precipitate was prepared by adding about 50 mM triethylamine to a 1 M solution of $VOSO_4$ and allowed to react for about ten minutes. This turned the blue vanadyl solution to a turbid slightly gelatinous brownish dispersion that was then collected by centrifugation. The precipitate was resuspended in 18 MΩ water and re-centrifuged. The washing was repeated and the final precipitate resuspended to approximately 10 mg/ml in water. A few µl of the dispersed precipitate was then applied to a 1000 Å thick silicon nitride window and dried in air.

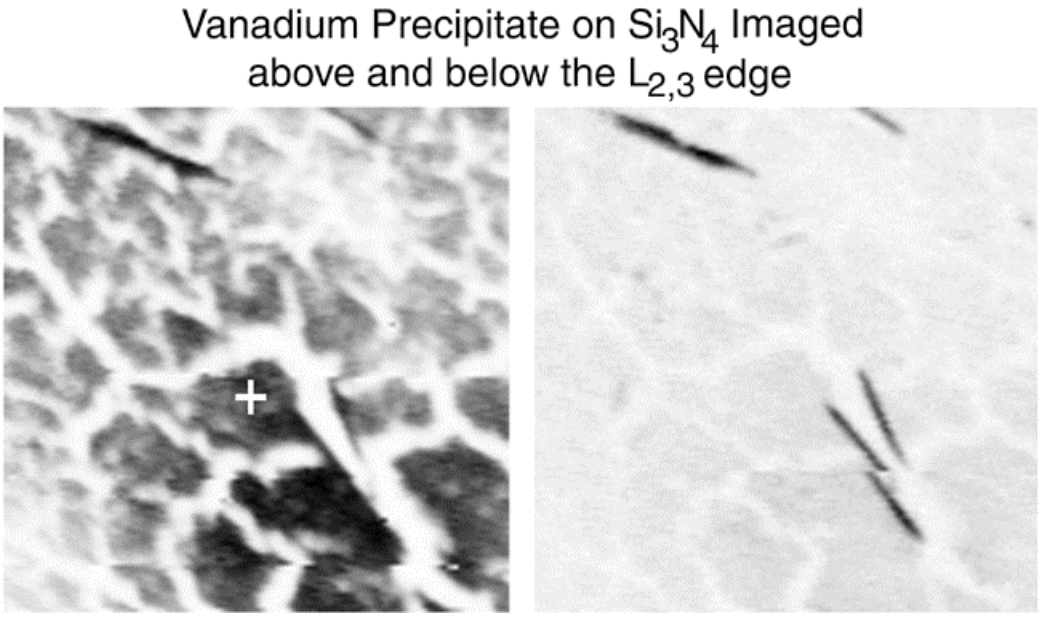
Cells. NIH 3T3 mouse fibroblasts were cultured in Dulbelco modified Eagles medium containing 10% calf serum (Gibco) at 37°C with 5% CO_2. Specimen substrates consisted of 1000 Å thick silicon nitride windows (3.5x3.5 mm) etched in 100 µm thick 12x12 mm silicon squares. Fibronectin coating was found to greatly improve cell adhesion to silicon nitride windows and was used routinely [5]. In either case, substrates were placed in petri dishes, covered with media, then layered with cells and cultured for 1 to 3 days until a convenient but subconfluent density was obtained. Fixed cell specimens were prepared by first rinsing off the culture media with phosphate buffered saline (PBS) pH 7.4, permeabilized for 30 seconds with 0.5% Triton X-100 in PBS, then fixed with a solution containing 4% paraformaldehyde, 0.2% glutaraldehyde, 0.5% Triton X-100, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, and 10 mM phosphate at pH 7.4 for 1 hr. Specimens were subsequently stored in PBS at 4°C until ready for treatment with vanadium. Fixed cell specimens to be labeled

with vanadium were first rinsed with 150 mM NaCl then incubated for several hours with 1 M $VOSO_4$. After the vanadyl incubation the cell specimens were rinsed several times with 18 MΩ water. In some samples, after an hour of vanadyl incubation, 10 to 20 mM triethylamine was added inducing hydrolysis of the vanadyl solution. After an additional 10 minute incubation, the specimens were rinsed with water. All vanadium treated silicon nitride cell samples were sandwiched with an opposing silicon nitride window and kept hydrated. All cell imaging was done with XM-1.

RESULTS

Figure 1 compares images taken with the ALS 7.0.1 STXM of the precipitated vanadium hydrated oxide above and below the L-edge resonances. The increase in x-ray absorbance at the higher energy is dramatic attaining 99% reduction of the transmitted signals in thicker areas of the film. Fig. 2 shows the absorption spectra of the precipitate taken with the STXM (at the crosshairs) and a similar spectrum taken with the CCD XM-1 imaging microscope of an aqueous 1 M $VOSO_4$ solution. It can be seen from both spectra that a 3.5-4 eV increase from just below the L-edge resonance (point of maximum transmission), results in about 90% of the full signal decrease corresponding to an optical density of 1.0. A very rough estimate can be made for the attenuation length for vanadium at the L resonance lines by using the signal at 527.5 eV to estimate the thickness of the $VOSO_4$ solution sampled in the XM-1 spectra. Using the tabulated mass absorption coefficients [7], we obtain an average estimate of 2 μm thickness. Using this value and the vanadium concentration of 0.051 gm/ml, we estimate an attenuation length of ~30 nm at the first vanadium L-edge resonance. This value is reasonably consistent with the vanadium attenuation length of 97 nm just past resonance [7].

FIGURE 1. The figure below shows STXM images of precipitated vanadium hydrated oxide taken above and below vanadium $L_{2,3}$ edges (128x128x0.3 μm per pixel). Cross hairs in left image indicate spot used to take spectra in Fig. 2 (left)



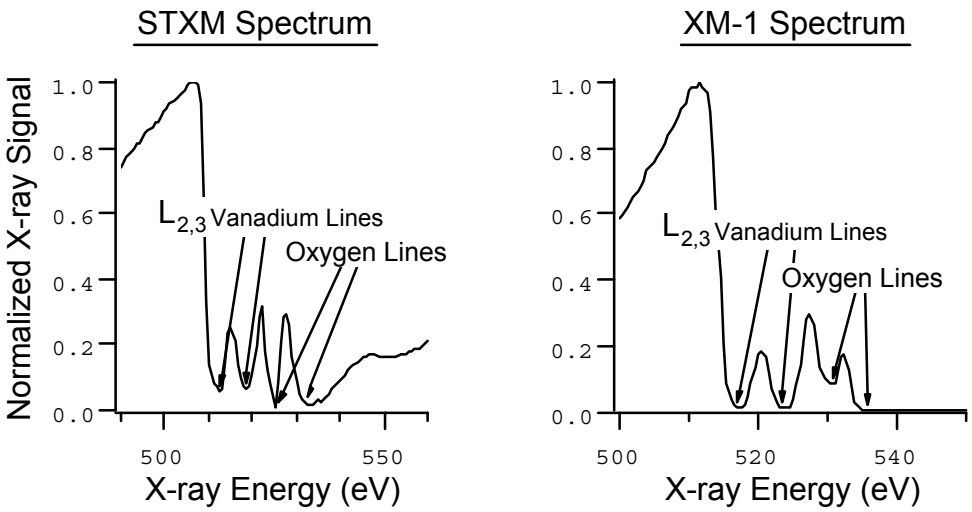
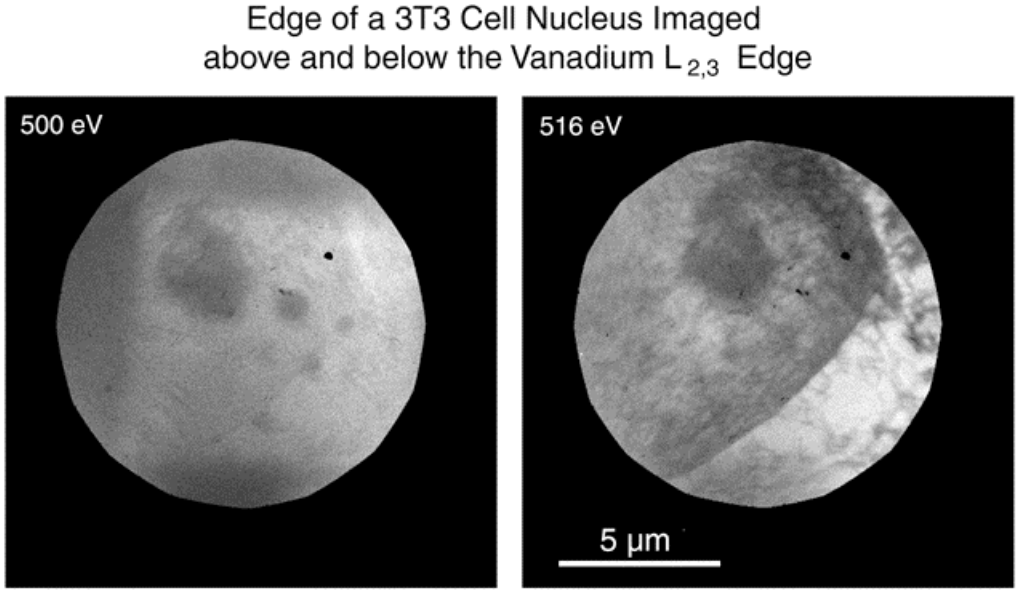


FIGURE 2. The figure above on the left shows the STXM spectrum obtained from the spot marked by crosshairs in Fig. 1. The right hand figure shows a similar spectrum obtained using XM-1 of a 1 M solution of $VOSO_4$.

The figures below show examples of NIH-3T3 fibroblasts incubated with vanadyl sulfate as discussed in METHODS. The vanadyl ion is divalent and as a consequence tends to bind strongly to negatively charged groups common to cell proteins, membranes, and nucleic acids. Figure 3 illustrates a striking increase in contrast of the cell nucleus when imaged at 516 eV compared to 500 eV. At the lower energy, the border of the nucleus is virtually imperceptible, whereas at the higher energy the entire nucleus stands out clearly. Fig. 4 shows a region dense in lamellopodia first imaged at 516 eV, 500 eV, then again at 516 eV.

FIGURE 3. 3T3 cells incubated with $VOSO_4$ then imaged using the CCD imaging microscope XM-1.



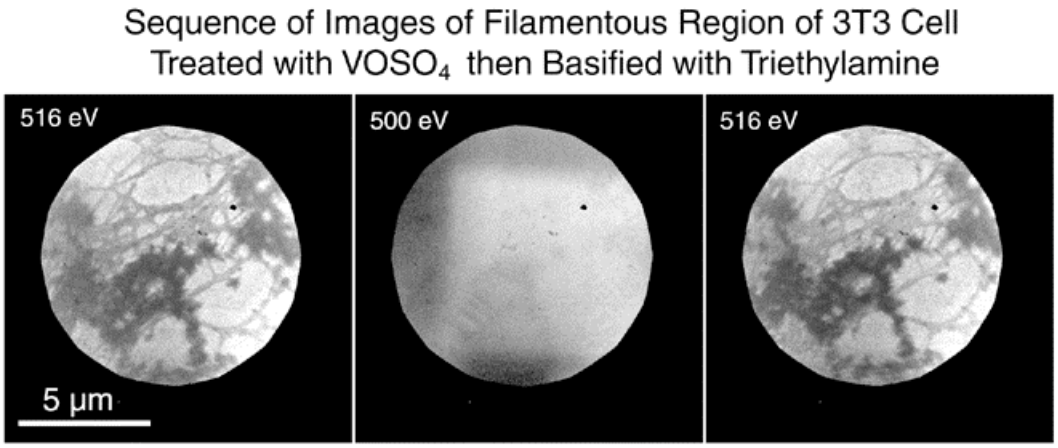


FIGURE 4. 3T3 cells incubated with $VOSO_4$ then basified with triethylamine. Images were acquired from left to right using XM-1. Dark dense material most likely to be hydrated vanadium oxide crystallites.

Figure 5 shows the contrast enhancement provided by the vanadyl binding to the cytoplasmic edge of a fixed cell. In this pair of images, the change in energy is a mere 3 eV. Nonetheless, the contrast increases at the cell boundary from 29% to 57% (cross, left image).

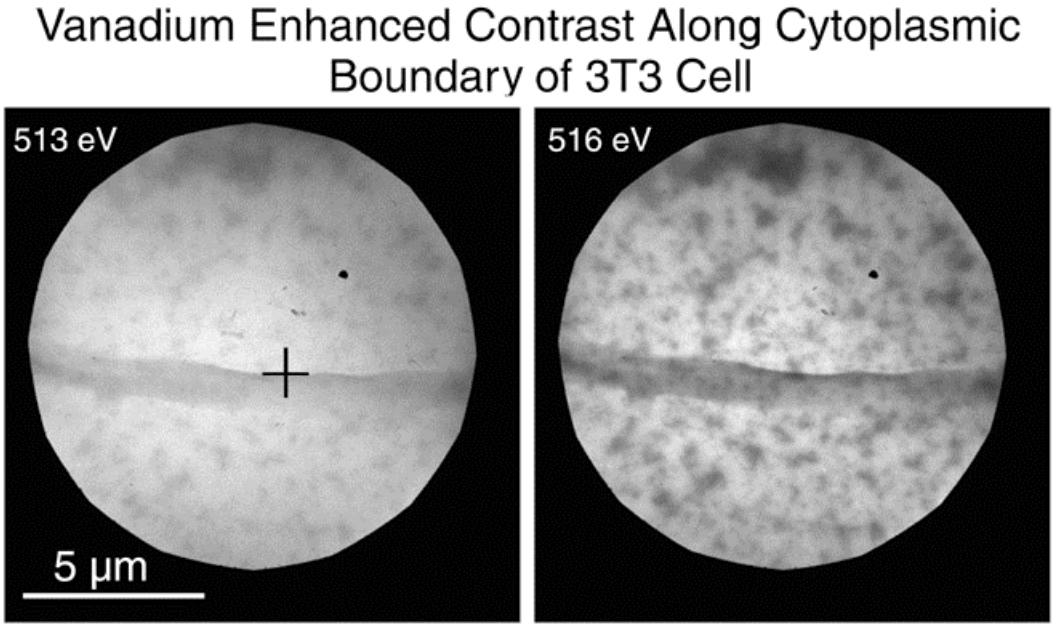


FIGURE 5. 3T3 cells incubated with $VOSO_4$ and imaged along cytoplasmic edge of lamellopodia. Cross (left image) indicates point used to compare relative contrasts at 513 and 516 eV.

CONCLUSION

It is clear from the data obtained, that vanadium offers the possibility of a second contrast probe to complement silver enhanced gold labeling with CCD-imaging x-ray microscopes. Based on the spectral data collected, the resonant absorption at the L-edge should make it practical to specifically image vanadium particles that are 20 nm or smaller. For labeling purposes, vanadium nanocrystals should be 5 nm or smaller to insure adequate cell penetration. By careful choice of hydrolytic conditions that control vanadium oxide formation, it should be possible to make these small crystals, which can then be derivatized by conventional silane chemistry providing linkers to biological recognition molecules such as antibodies and avidin.

ACKNOWLEDGEMENTS

This paper is dedicated to our dear friend and colleague Werner Meyer-Ilse whose untimely death is a tragic loss for us all.

The authors further acknowledge the support by the US. Department of Energy, Office of Health and Environmental Research, and the Office of Basic Energy Sciences under contract number DE-AC03-76SF00098.

REFERENCES

1. Kirz, J., Jacobsen, C. and Howells, M. (1995). Soft x-ray microscopes and their biological applications. *Quarterly Reviews of Biophysics* 28(1): 33-130.

2. Chapman, H. N., Fu, J., Jacobsen, C., and Williams, S. (1996) Dark field x-ray microscopy of immunogold-labeled cells. *J. Microscopy Soc. Am*. 2(2): 53-62

3. Jacobsen C; Lindaas S; Williams S; Zhang X. (1993) Scanning luminescence x-ray microscopy - imaging fluorescence dyes at suboptical resolution. *J. Microscopy* 172: 121-129.

4. M. M. Moronne, C. Larabell, P.R. Selvin, and A. Irtel von Brenndorff (1994), Development of fluorescent probes for x-ray microscopy. *Microscopy Society of America* 52:48-49.

5. Moronne, M.M. (1999), Development of x-ray excitable luminescent probes for high resolution scanning x-ray microscopy. *J. Ultramicroscopy*, 77:23-36

6. T. Warwick, H. Ade, S. Cerasari, J. Denlinger, K. Franck, A. Garcia, S. Hayakawa, A P. Hitchcock, J. Kikuma, S. Klinger, J. Kortright, G. Meigs, G. Morisson, M. Moronne, S. Myneni, E. Rightor, E. Rotenberg, S. Seal, H-J. Shin, W.F Steele, T. Tyliszczak, and B. P. Tonner (1998). A scanning transmission x-ray microscope for materials science spectromicroscopy at the Advanced Light Source. *Rev. Sci. Instr.* 69(8): 2964-73

7. B.L. Henke, E.M. Gullikson, and J.C. Davis (1993). X-ray interactions: photoabsorption, scattering, transmission, and reflection at E=50-30000 eV, Z=1-92, *Atomic Data and Nuclear Data Table*s, 54 (2): 181-342.